MICRORNA PROFILING OF MONOCYTE TO OSTEOCLAST DIFFERENTIATION REVEALS MRNA TARGETS LINKED TO OSTEOCLASTOGENESIS

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ABSTRACT

Katherine A. Guilfoyle: MiRNA Profiling of Monocyte to Osteoclast Differentiation Reveals mRNA Targets Linked to Osteoclastogenesis
(Under the direction of Salvador Nares)

OBJECTIVES: To investigate differentially expressed microRNAs (miRNAs) and predicted target genes over 12 days of osteoclastogenesis. METHODS: Leukophoresed buffy coats were obtained from healthy human donors (N=4) and CD14+ monocytes isolated. Osteoclastogenesis was induced by culturing cells with sRANKL and M-CSF. Freshly isolated monocytes and monocytes cultured with M-CSF alone were utilized as controls. At 4 time points, RNA was isolated and miRNA levels interrogated using microarrays (N=36.) MiRNA-mRNA target interactions for differentially expressed miRNAs were investigated using publically available algorithms. RESULTS: MiRNA profiling revealed conserved and differentially expressed miRNAs; both novel and previously reported in osteoclastogenesis. Several differentially expressed miRNAs shared predicted mRNA targets and given targets were targeted by multiple miRNAs. CONCLUSIONS: MiRNA profiling osteoclastogenesis identified previously associated miRNAs and novel miRNAs not previously described in osteoclast differentiation. *In-silico* analysis reveals potential mRNA targets serving as candidates for future investigation, with possible future implications in human diseases.

TABLE OF CONTENTS

LIST OF TABLES……..……iv

LIST OF FIGURES……..…..vi

LIST OF ABBREVIATIONS……...……vii

CHAPTER 1: A REVIEW OF OSTEOCLASTOGENESIS AND THE ROLE OF MICRORNA ON OSTEOCLAST DIFFERENTIATION AND FUNCTION...……..1

Introduction…….....1

Differentiation……..……2

Osteoclast Action…….……3

Disease Pathogenesis…….……..4

MicroRNA Action in Osteoclastogenesis…….....9

Conclusion……12

CHAPTER 2: MIRNA PROFILING OF MONOCYTE TO OSTEOCLAST DIFFERENTIATION REVEALS MRNA TARGETS LINKED TO OSTEOCLASTOGENESIS.....14

Introduction……14

Materials and Methods……..……..17

Isolation of Monocytes and Differentiation into Macrophages and Osteoclasts……...……17

Flow Cytometry…….17

TRAP Staining……..…….19

Osteolyse™ Assay……...……..19

Cell Viability……..……19

Total RNA Isolation…….……..20

MicroRNA Arrays…….……20

Statistical Analysis…….……20

Network Analysis……...……21

Target Selection……...……..21

Results……...…….22

Discussion…….…….27

FIGURES AND TABLES…….……32

REFERENCES……..……48

LIST OF FIGURES

Figure 1: Phenotyping data……32

Figure 2: TRAP stain data……..……33

Figure 3: Microscopy data…….……34

Figure 4: Representative Osteolyse™ results……...…….35

Table 1: MiRNAs differentially expressed after Benjamini Hochberg Adjustment….…….36

Figure 5: MiRNAs differentially expressed in M-CSF + sRANKL cells……...……...37

Table 2: MiRNAs differentially expressed after Bonferroni correction……38

Figure 6: Signal intensity over time……..…….39

Figure 7: Pathway analysis……40

Table 3: Target gene function from pathway analysis……..….41

Table 4: MiRNAs differentially expressed and osteoclast related gene targets……...…….44

Figure 8: Signal intensity in up-regulated miRNAs…….…….46

Figure 9: Signal intensity in down-regulated miRNAs……..47

LIST OF ABBREVIATIONS

µL	Microliter
ANOVA	Analysis of variance
APCs	Antigen presenting cells
Ago2	Argonaute RISC catalytic component 2
ASOs	Antisense oligonucleotides
BMMs	Bone Marrow-derived Monocyte/Macrophages
C5a	Complement component 5a
CATK	Cathepsin K
CD14	Cluster of Differentiation 14
CD14	Cluster of Differentiation 14 positive
CD28	Cluster of Differentiation 28
CD80	Cluster of Differentiation 80
CD86	Cluster of Differentiation 86
CDw93	Cluster of Differentiation 93
C1qRp	C1q Receptor for Phagocytosis Enhancement
CO_2	Carbon dioxide
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DGCR8	DGCR8 microprocessor complex unit
dH_2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
ETS	E26
FACS	Fluorescence-activated Cell Sorting
FBS	Fetal bovine serum
FcR	Fc receptor

FITC	Fluorescein isothiocyanate
FOSL2	Fos-like antigen 2
Fra-1	Fos-related Antigen
IFN-β	Interferon Beta
IGF-1	Insulin-like Growth Factor-1
IgG	Immunoglobulin G
IL-1	Interleukin 1
logFc	Log fold change
LPS	Lipopolysaccharide
M	Molar
MAPK	Mitogen-activated Protein Kinase
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony-stimulating Factor
M-CSFR	Macrophage Colony-stimulating Factor Receptor
MIP-1α	Macrophage Inflammatory Protein 1 alpha
MITF	Microphthalmia-associated Transcription Factor
miRNAs	MicroRNA
mL	Mililiter
MMPs	Matrix metalloproteinases
NFATc1	Nuclear Factor of Activated T-cells Cytoplasmic 1
NFI-A	Nuclear Factor 1-A
NF-κβ	Nuclear Factor Kappa Beta
Ng	Nanogram
Nm	Nanometer
OPG	Osteoprotegrin

PBS	Phosphate buffered saline
PDCD4	Programmed Cell Death 4
PFA	Paraformaldehyde
PI3K	Phosphoinositide 3-kinase
PTH	Parathyroid hormone
RANK	Receptor for Activation of Nuclear Factor Kappa Beta
RANKL	Receptor for Activation of Nuclear Factor Kappa Beta Ligand
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SDF-1α	Stromal Cell-derived Factor 1 alpha
SOCS1	Suppressor of Cytokine Signaling 1
sRANKL	Soluble Receptor for Activation of Nuclear Factor Kappa Beta Ligand
TGFβR1	Transforming growth factor, beta receptor
TIMPs	Tissue inhibitors of metalloproteinase
TNF-α	Tumor necrosis factor-alpha
TRAF6	Tumor Necrosis Factor Receptor-associated Factor 6
TRANKL	TRAF Family Member-associated Nuclear Factor Kappa Beta Ligand
TRAP	Tartrate resistant acid phosphatase
UTR	Untranslated region

A REVIEW OF OSTEOCLASTOGENESIS AND THE ROLE OF MICRORNA ON OSTEOCLAST DIFFERENTIATIONAND FUNCTION

Introduction

Osteoclasts are multinucleated cells derived from a myeloid lineage, which, through the process of osteoclastogenesis, differentiate from their hematopoietic precursors; monocytes/macrophages.[1] Osteoclasts were originally confirmed to have a hematopoietic origin when osteopetrotic mice were apparently cured following injection of normal spleen cells[2] and the hematopoietic origin was later confirmed in humans following a brother-to-sister bone marrow transplant.[3] These observations that the presence of spleen or bone marrow cells together with stromal cells in fact yielded osteoclasts, was key in our understanding of osteoclastogenesis.[4] These cells play a key role in the remodeling of bone, and thus the regulation of skeletal mass as resorptive cells; they are unique cells in that they resorb bone.[5] When imbalance occurs between the resorptive action of osteoclasts and the bone-forming action of osteoblasts, pathological conditions, such as osteoporosis may exist. The individual with this disorder may experience pathological bone fractures due to a reduction in the density of skeletal mass. A deep understanding of the osteoclast; how it is formed and its action, as well as the regulation of these processes is important in acquiring an understanding of such disorders, and may impact future therapeutic goals. MicroRNAs (miRNAs) influence the differentiation process of osteoclasts on a genetic level. Expression profiles obtained through miRNA analysis, showing which miRNAs, and thus, which likely target genes are up/down regulated throughout osteoclastogenesis allows for a deeper understanding of the differentiation process.

Differentiation

In 1990, Udagawa et al. noted that marrow stromal cells or osteoblast progeny must be present for the *in vitro* maturation of macrophages to osteoclasts to occur.[1] Two molecules are expressed that are necessary to promote osteoclastogenesis: macrophage colony-stimulating factor (M-CSF), a polypeptide growth factor, and receptor for activation of nuclear factor kappa beta (NF-κβ) (RANK) ligand (RANKL), a TNF-related cytokine.[6, 7] M-CSF promotes the proliferation and survival of osteoclast progenitor cells, while RANKL prompts the differentiation through the osteoclast lineage and acts as both an activating and survival factor for mature osteoclasts.[8-10] M-CSF[1] binds its receptor c-FMs on osteoclast precursors and signals the survival and proliferation of the cells. For osteoclastogenesis to occur, osteoclast precursors must be in contact with stromal cells or osteoblasts, [1] as RANK, found on osteoclasts and their precursors, interacts with RANKL, which resides on stromal cells.[7] RANK-RANKL binding is essential for bone remodeling, as shown in mice deficient in either RANK or RANKL, sharing the same phenotype.[11-14] RANK-RANKL interaction stimulates downstream signaling cascades, including mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) and NF-κβ.[10, 15] RANKL is able to activate mature osteoclasts *in vitro* in a dose-dependent manner and activate pre-existing osteoclasts *in vivo*; rapidly leading to bone resorption.[11, 16, 17] It leads to induction of osteoclastogenic transcription factors c-Fos, fos-related antigen 1 (Fra-1) and cytoplasmic, calcineurin-dependent nuclear factor of activated T-cells cytoplasmic 1 (NFATc1.)[10, 18-21] M-CSF and RANKL are both necessary for the promotion of osteoclastogenesis, and they are needed to induce expression of genes that encode tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and the B_3-integrin[7]

so that mature osteoclasts may develop.[11] Simon et al. noted in 1997 that overexpression of osteoprotegrin (OPG) blunts osteoclastogenesis in mice, and those animals lacking OPG were noted to have accelerated osteoclastogenesis and thus developed osteoporosis[22]. OPG, a secreted TNFR-related protein which regulates density and mass of bone, [22, 23] is a "decoy" receptor, which competes with RANK on osteoclasts and their precursors for RANKL.[7] The amount of bone resorbed is dictated by a shift in balance between RANKL, which stimulates osteoclastogenesis; and OPG, the inhibitor.[24] As further evidence that the protein complex, NF-κβ plays a key role in osteoclastogenesis, mice that lack the p50 and p52 subunits of the complex develop osteopetrosis due to the inability of osteoclasts to differentiate from macrophages.[25] The survival of the mature osteoclast is regulated by hormones and cytokines[11, 26] and it has been shown that RANKL and interleukin (IL)-1 increase survival time both *in vitro* and *in vivo*; possibly due to their ability to induce NF-κβ.[27, 28]

Osteoclast Action

Osteoclasts are unique, multinucleated, bone resorptive cells, which degrade bone tissue in a multistep process. With its "ruffled membrane," the osteoclast is able to polarize on bone, forming a resorptive organelle, which is only present when the cell is attached to bone tissue.[29] Polarization of the osteoclast cell body occurs in response to RANK activation by RANKL[16] leading to structural changes, including the rearrangement of the actin cytoskeleton and the formation of a tight junction, a sealed compartment between the bone and basal membrane of the cell.[11, 16] The export of hydrogen ions leads to the acidification of this "vacuole" [11, 30] and lytic enzymes, such as tartrate resistant acid phosphatase (TRAP) and pro-cathepsin K (pro-CATK) are also released into the pit; or Howship's lacunae.[11] As the osteoclast erodes bone, degradation

products are produced, including fragments of collagen and solubilized calcium and phosphate; which after processing by the osteoclast, are released into circulation.[11]

Several genes or loci regulate osteoclastogenesis and the activation of the mature osteoclast in either positive or negative fashions. This has been shown through both naturally occurring mutations and targeted knockout mutations in animals and humans.[11, 31, 32] Disruption of gene activity may lead to osteopetrosis or osteopenic conditions. The effects of genes may be exerted at various steps of osteoclast development and activation; for example, *PU.1* and *op/CSF-1* act during the formation and survival of osteoclast precursor cells, while other genes, such as *RANK*, *p50/p52 rel* and *fos* affect the differentiation of these precursor cells towards a multinucleated cell,[11] as discussed previously. *PU.1* encodes a transcription factor of the E26 or ETS family and activates gene expression during myeloid cell development, while op/CSF-1, encodes a key cytokine to macrophage production, differentiation and function. Other genes, such as *src*, *oc/Tcirg* and *CATK* affect the actions of the mature osteoclast, such as its ability to adhere to bone and perform lytic functions[11].

Disease Pathogenesis

In health and in young adults, the destruction and formation of bone is balanced, with osteoclasts resorbing bone in approximately 3 weeks per site, and osteoblasts forming new bone tissue in approximately 3-4 months.[29] This process is normal and necessary for bone to carry out its supportive and mechanical functions. The influences by mechanical use[33, 34] as well as the likely influence of central homeostatic factors[35] help guide the resorption and rebuilding of bone, to maintain a steady state.[36] A majority of skeletal diseases occurring in adults are due to an excess of osteoclast action, which leads to an imbalance in the bone remodeling process, such

that resorption is increased.[36] Such skeletal diseases include osteoporosis, periodontal disease, rheumatoid arthritis, multiple myeloma and metastatic cancers.[11]

Osteoporosis is a systemic skeletal disease which is characterized by a decrease in bone mass, leading to increased bone fragility and risk of fracture.[37] Approximately 70 million individuals worldwide are at risk, and bone fractures in those with osteoporosis may potentially be life-threatening occurrences.[11] The peak bone mass is reached in the third decade of life; and although a 'physiologic' age-related change, the mass decreases approximately 0.5% per year.[38, 39] Several factors, such as genetics, nutrition and physical activity increase the risk of fractures along with age-related bone loss; however, this bone loss alone does not necessarily predispose one to fractures.[39, 40] Two types of osteoporosis have been classified; due to pathophysiology and regions affected: post-menopausal osteoporosis and senile osteoporosis. Post-menopausal osteoporosis is due to decreased estrogen levels in post-menopausal women, it mainly affects trabecular bone, and is associated with vertebral and wrist fractures.[39, 41] Estrogen helps to regulate the activity of osteoclasts, and so, low estrogen levels during menopause facilitate the differentiation and activity of osteoclasts. Simultaneously, available osteoblast numbers, as well as osteoblastic activity is decreased due to hormone-independent mechanisms.[39, 42] Senile osteoporosis occurs at a similar rate in both sexes, usually after the sixth decade of life, and affects cortical bone most frequently; predisposing individuals to hip fracture.[39, 43] In senile osteoporosis; osteoclast activity levels are high and osteoblast availability/activity is reduced due to decreased osteoblast differentiation from mesenchymal cells, which have undergone a shift to differentiate into adipocytes; an increasing marrow fat infiltration that affects survival and function of osteoblasts; and high levels of osteoblast apoptosis. These mechanisms, which occur in both males and females, affect the aging, osteoporotic bone by reducing its capacity to repair

areas that have weakened; and so, leaving it predisposed to the occurrence of fracture.[39, 43, 44] Changes in cell-to-cell communication which occur with age, also affect osteoporotic bone, making it more fracture-prone; the most common changes being an increase in RANKL, a decrease in OPG[45] and an increased level of sclerostin,[46] which is released by osteocytes and inhibits osteoblast activity.[39]

Osteopetrosis is condition which is characterized by abnormally high levels of mineralized bone and cartilage; and occurs when the development and/or function of mature osteoclasts is negatively affected.[11] There are either a decreased number of osteoclasts or the cells are defective in function.[47] The condition was first reported in 1904 by Albers-Schönberg, who described it as "marble bone disease."[47, 48] A variety of manifestations result from osteopetrosis, ranging from mild symptoms to death at an early age.[47] The bone marrow space may become crowded, leading to inability to produce red blood cells, white blood cells and platelets,[47, 49, 50] thus resulting in anemic conditions and chronic infections[47] due to these reduced blood cell counts. These effects upon the bone marrow are usually noted in the autosomal recessive form of osteopetrosis; with other manifestations including hearing loss, blindness, macrocephaly hepatosplenomegaly, short stature, abnormalities in the dentition and failure to thrive Children affected by this condition have a 98-99% mortality rate by the age of 10 years.[47, 49, 50] An autosomal dominant form exists, with less severe manifestations and a typically later diagnosis. Loss of vision with optic atrophy is a common neurological finding, occurring due to bony encroachment upon the optic nerve[47, 49, 51] Pathological bone fractures occur as well, and this finding typically leads to the diagnosis in later childhood or adulthood.[47, 49, 51] One must keep in mind that the excess bone found in osteopetrosis is due to lack of osteoclastic action, so

the bone present is not of the same quality as that formed through normal resorption and remodeling processes. Fractures occur due to the bone quality, not quantity.

Periodontal disease and peri-implant bone loss are affected by the actions of osteoclasts. In periodontal disease, periodontal pathogens trigger inflammatory and immune responses, which affect the clinical outcome of the condition; such as loss of alveolar bone and connective tissue attachment. Inflammatory mediators trigger the bone resorption and proteases which degrade the extracellular matrix, leading to tissue destruction.[52-56] To this day, much remains unknown with regards to the activation of osteoclasts as it relates to periodontal disease pathology, and a better understanding is key in the evolution of new treatments for periodontitis.[57] Garlet et al. 2004 investigated expression patterns of mRNAs which encoded for matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase (TIMPs), RANKL and OPG by real-time polymerase chain reaction (RT-PCR) and correlated patterns with cytokine expression within gingival biopsies from healthy subjects and those with chronic and aggressive periodontitis. The study noted that the aggressive and chronic periodontitis groups displayed similar levels of expression of MMPs, RANKL and TNF-α; however, the expression of TIMPs, OPG and IL-10 were higher in those with chronic periodontitis. The authors suggest that the higher expression of OPG in those with chronic periodontitis (compared to those with aggressive periodontitis) could perhaps partially control the alveolar bone loss driven by RANKL, reducing the progression rate and severity of this form of periodontal disease. Lower expression of OPG in aggressive periodontitis would then be a potential influence of more severe disease[56] with regard to progression rate of bone loss. The study concludes that the pattern of cytokines expressed in the two disease forms affects the progression and severity of disease through effects upon MMP/TIMP and RANKL/OPG balance in the tissues.[56] Niwa et al. recently examined the

effects of chemoattractants in periodontitis tissues upon osteoclast chemotaxis using the EZ-TAXIScan™ method, which measures the chemotactic speed and angle through a concentration gradient between a glass plate and thin microfabricated silicon chip, or channel.[57] The chemotactic responses of day 3 and 6 osteoclasts to several chemoattractants expressed in periodontitis tissue were measured, including MCP-1, MIP-1α, RANTES, SDF-1α and C5a. The highest chemotactic response of day 6 osteoclasts, with regards to chemotactic speed, direct migration and percentage of chemotactic cells, was noted to be towards C5a. Furthermore, the speed of chemotaxis towards C5a increased from day 3 to day 6; these findings were in agreement with Ignatius et al., who found that the expression of the C5a receptor increases throughout osteoclast differentiation.[57, 58] Previous studies have shown that *Porphyromonas gingivalis* produces Arginine-specific protease gingipain, which results in increased C5a production.[57, 59, 60] C5a is reported to inhibit neutrophil immune activation when within a range of 10-100nm[61-63] and Niwa et al. reported that 100nm of C5a induced the highest chemotactic response for the day 6 osteoclasts. The authors state that a *P. gingivalis*-induced increase in C5a must play a role in the progression of periodontitis by suppressing the immune response of neutrophils while increasing the chemotaxis of osteoclasts.[57] Lastly, the Niwa et al. study found that H-8135, a selective C5a receptor antagonist inhibits both day 3 and 6 osteoclast chemotaxis towards C5a. Antagonistic activity inhibiting the recruitment of osteoclasts would thus inhibit the bone resorption by osteoclasts;[57] fueling interest in such antagonists for future therapeutic methods.

A recent study by Kadkhodazadeh et al. investigated the possibility of the RANKL gene being a genetic determinant for peri-implantitis.[64, 65] The study found that a particular genotype (CT) of the rs9533156 TANKL gene polymorphism was significantly associated with peri-

implantitis in an Iranian population, and suggested that it could be considered as a genetic determinant for peri-implantitis.[65] Rakic et al. studied the levels of biomarkers in gingival crevicular fluid associated with osteoclastogenesis (sRANKL, RANK and OPG) in subjects with peri-implantitis and compared them to levels in those with healthy peri-implant sites and severe chronic periodontitis sites. When compared to healthy sites, levels of sRANKL, RANK and OPG were all significantly higher in peri-implantitis sites, although the sRANKL/OPG ratios were not statistically significantly different. When compared to periodontitis sites, RANK was significantly higher in peri-implantitis sites; however, sRANKL and the sRANKL/OPG ratio were significantly higher in periodontitis sites. The authors concluded that while there were differences in the patterns of the biomarkers in comparison with periodontitis sites, all three were associated with peri-implant tissue destruction.[66]

MicroRNA Action in Osteoclastogenesis

As previously discussed in this review, the regulation of osteoclastogenesis is influenced by M-CSF and RANKL, as well as sex steroids, parathyroid hormone (PTH), vitamin D, insulin-like growth factor-1 (IGF-1), calcitonin, prostaglandins[5, 67] and transcription factors, such as c-fos, PU.1, NFκβ (p50 and p52 subunits) and nuclear factor of activated T cells cytoplasmic 1 (NFATc1).[18, 21, 68-71] Recent studies have shown that microRNAs (miRNAs) are also necessary for osteoclastogenesis, as they influence osteoclast differentiation, function and survival.[71] Gene silencing studies of DGCR8, Dicer and Ago2 have verified the role of miRNAs in osteoclast differentiation and function; as these genes are needed for miRNA homeostasis.[72-74]

MiRNAs are small, non-coding, single-stranded RNAs that measure approximately 19-25 nucleotides in length, and which have been found to regulate several pathways in the cell cycle, including differentiation, proliferation, apoptosis and tumorigenesis.[75] MiRNAs regulate the

expression of an estimated 8000 genes, which amounts to approximately 30% of the human genome.[76-78] Each miRNA may suppress multiple genes; approximately 200, on average; and a single mRNA can be targeted by multiple miRNAs.[77, 79-81] In eukaryotes, miRNAs form imperfect hybrids, as there is only partial complementarity, with the 3' untranslated region (UTR) sequences of target mRNAs, enabling regulation. As a result, translational repression and/or mRNA degradation occurs.[82] MiRNAs have been shown to be associated with actively translating mRNA[83] and several mechanisms may contribute to miRNA-mediated regulation of genes; which may vary depending on the gene, cell and condition.[84]

Particular miRNAs have been identified as influential in osteoblast differentiation and bone formation; while there is limited knowledge regarding the regulatory role of miRNAs in osteoclastogenesis. MiRNAs involved in osteoblast differentiation and bone formation include, but are not limited to[71] miR-125b,[85] miR-26a,[86] miR-133 and miR-135,[87] miR-204/211,[88] miR-29a,[89] miR-141 and miR-200a,[90] miR-206,[91] miR-29b,[92] miR-210,[93] miR-196a[94] and miR-2861.[95]

While miRNA function in osteoclastogenesis is not yet completely understood; several miRNAs have been noted to play a role. Sugatani et al. studied the miRNA signature of RANKL-induced osteoclastogenesis and showed that 38 miRNAs were elevated and 33 were downregulated.[96] MiRNAs with a known role in the differentiation and function of osteoclasts include miR-21, miR-223, miR-155 and miR-146a. The overexpression of miR-21 leads to promotion of c-Fos (a transcription factor which is critical in osteoclastogenesis) through the down-regulation of programmed cell death 4 (PDCD4) protein levels, via repression removal from c-Fos.[97] Thus, a positive feedback loop exists between miR-21, c-Fos and PDCD4; related to regulation of osteoclastogenesis.[96] Sugatani et al. noted that miR-21 was robustly stimulated in

RANKL-induced osteoclastogenesis.[71, 97] MiR-223 has been shown to stimulate the expression of macrophage colony-stimulating factor receptor (M-CSFR); however, overexpression of pre-miR-223 has been found to block the formation of osteoclasts.[98] Induction of PU.1 by M-CSF, will stimulate the expression of this miRNA, which thus down regulates NFI-A, which is needed to up-regulate M-CSFR levels. Increased expression levels of PU.1, microphthalmia-associated transcription factor (MITF) and c-Fos are induced by M-CSF and [99]RANKL through the up-regulation of M-CSFR and RANK.[73]

Sugatani et al. used chemically modified antisense oligonucleotides (ASOs), also known as antagomirs, which are complementary to specific miRNAs to investigate how essential the roles of miR-21 and mir-223 were to osteoclastogenesis. Antagomirs were developed for investigative and therapeutic approaches to silencing miRNAs *in vivo*. They are cholesterol-conjugated, single-stranded RNA analogs, which can transiently interfere with miRNA.[71, 100-102] The ability of antagomirs as specific silencers of endogenous miRNAs have made them useful in investigating gene functions both *in vitro* and *in vivo*[71, 99, 103] and in clinical trials via antisense therapeutics.[71, 104, 105] Sugatani et al. transduced bone marrow-derived monocyte/macrophage precursors (BMMs) with antisense miR-21-containing lentivirus and found extreme up-regulation of PDCD4, which is targeted by miR-21. Remarkable down-regulation of RANKL-induction of c-Fos phosphorylation, and expression of NFATc1 and cathepsin K protein were noted in cells with reduced levels of miR-21 due to the silencing effect. Thus, normal expression of miR-21 is relevant to osteoclastogenesis.[71, 97] The same group investigated the role of miR-223 in osteoclastogenesis using antagomirs. It was found that the inhibition of miR-223 induced down-regulation of TRAP-positive osteoclast formation. Additionally, NFI-A levels were up-

regulated, which resulted in a decrease of M-CSFR levels. It was suggested that miR-223 is essential for osteoclastogenesis as well, via expression of M-CSFR.[71, 73]

MiR-155 and miR-146a have been found to significantly down-regulate MITF and NFATc1, respectively, both of which play important roles in osteoclast differentiation.[106, 107] Zhang et al. studied miR-155 targeting of suppressor of cytokine signaling 1 (SOCS1) and MITF in a murine model. It has been previously shown that miR-155 is induced by IFN-β and that IFN-β suppresses the differentiation of osteoclasts.[10, 107, 108] SOCS1 and MITF promote osteoclast differentiation by inhibiting IFN-β downstream signaling and by cooperating with NFATc1,[10, 11, 109] respectively.[10] Zhang et al. found that mir-155, which is an IFN-β inducible miRNA, can target SOCS1 and MITF and mediate the suppressive effect of IFN-β on osteoclastogenesis.[10]

The effects of miR-146a overexpression upon osteoclastogenesis were studied by Nakasa et al. in 2001. This study showed in a murine model that miR-146a led to a significant down-regulation of c-Jun, NFATc1, PU.1 and TRAP. Results further suggested that one mechanism of osteoclastogenesis suppression was the down-regulation of TNF receptor-associated factor 6, (TRAF6), a validated target of miR-146a[110] and critical signaling molecule in RANKL-mediated osteoclastogenesis.[111-113]

Conclusion

The differentiation of monocytes to multinucleated osteoclasts is a complex process affected by several different transcription factors and genes. As this review discusses, several pathological conditions arise from a lack of balance between the function of osteoblasts, forming bone and osteoclasts resorbing bone. A deeper understanding of the process of

osteoclastogenesis will enable further treatment modalities to be developed for individuals suffering from these diseases, and perhaps help in prevention someday. The small miRNA, only approximately 22 nucleotides in length, can have major impacts upon osteoclastogenesis and disease pathogenesis through the regulation of gene targets. Studies have identified several miRNAs that play roles in osteoblast and osteoclast differentiation and function; however, as miRNAs have been studied for only a short period of time thus far, further research into the identification and characterization of additional miRNAs is needed The future will likely hold vast and exciting discoveries through the investigation of miRNAs, which may deeply impact medical interventions.

MIRNA PROFILING OF MONOCYTE TO OSTEOCLAST DIFFERENTIATION REVEALS MRNA TARGETS LINKED TO OSTEOCLASTOGENESIS

Introduction

Osteoclastogenesis is the process by which bone resorptive cells, osteoclasts, differentiate from their hematopoietic precursors; monocytes and macrophages.[1] The role osteoclasts play in bone remodeling is important for the regulation of skeletal mass[5] and in keeping balance with the action of bone-forming cells, osteoblasts. In order for osteoclasts to differentiate from their precursors, two molecules must be expressed: macrophage colony stimulating factor (M-CSF), which is a polypeptide growth factor; and receptor for activation of nuclear factor kappa β (NF-κβ) (RANK) ligand (RANKL), which is a TNF-related cytokine.[6, 7] These molecules are necessary as M-CSF promotes both the proliferation and survival of osteoclast progenitors and RANKL prompts the differentiation along the osteoclast lineage, behaving as an activating and survival factor for mature osteoclasts.[8-10] RANK on the osteoclast must interact with RANKL, which resides on the stromal cells, for osteoclastogenesis to occur.[7] This interaction stimulates downstream signaling cascades, which include the mitogen-activated protein kinase (MAPK) pathway, phosphoinositide 3-kinase (PI3K) and NFκβ.[10, 15] RANKL induces osteoclastogenic transcription factors c-Fos, fos-related antigen 1 (Fra-1) and cytoplasmic, calcineurin-dependent nuclear factor of activated T-cells cytoplasmic 1 (NFATc1.)[10, 18-21] Together, M-CSF and RANKL promote osteoclastogenesis by also inducing the expression of genes that encode tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and the B_3-

integrin[7], enabling mature osteoclasts to develop.[11] These and many other molecules, including hormones and cytokines,[11, 26] do affect the differentiation and survival of the osteoclast.

Through genetic mutations, occurring both naturally and through targeted knockouts, several genes or loci have been found to regulate osteoclastogenesis, as well as the activation of the mature osteoclast.[11, 31, 32] Genes may exert their effects at various stages of osteoclast development and activation, such as the formation and survival of precursor cells, differentiation to osteoclasts, or survival of the mature osteoclast.[11] Disruption of gene activity may influence occurrence of pathological conditions. Conditions such as osteoporosis and osteopetrosis may occur when an imbalance exists between osteoclast and osteoblast activity. A deeper understanding of the process of osteoclastogenesis may help in one day developing new therapeutic or preventative interventions for such conditions. The study of microRNAs (miRNAs) may be an informative path to gaining better understanding of osteoclastogenesis and bone resorptive disease pathogenesis.

MiRNAs are small, non-coding, single-stranded RNAs measuring approximately 19-25 nucleotides in length and have been found to regulate several cellular pathways.[75] They have been found to regulate the expression of an estimated 8000 genes, or approximately 30% of the human genome.[76-78] In eukaryotes, miRNAs form imperfect hybrids with their targets, the 3' untranslated region (UTR) sequences of mRNAs.[82] With this partial complementarity, a miRNA may target and suppress multiple genes; approximately 200 on average; and a single mRNA may be targeted by multiple different miRNAs.[77, 79-81] MiRNAs have been shown to be necessary for osteoclastogenesis, as they influence the differentiation, function and survival of osteoclasts.[71]

Several miRNAs have been identified as being influential in osteoblast differentiation and bone formation[71], including, miR-125b[85], miR-26a[86], miR-133, miR-135[87], miR-204/211[88], miR-29a[89], miR-141, miR-200a[90], miR-206[91]miR-29b [92], miR-210[93], miR-196a[94] and miR-2861[95]. Fewer studies have investigated the role of miRNAs in osteoclastogenesis, although some have been identified. MiRNAs with a known role in the differentiation and function of osteoclasts include, but are not necessarily limited to miR-21, miR-223, miR-155 and miR-146a.

Mir-21 overexpression has been found to lead to the promotion of C-Fos[97], a key regulator in the osteoclast lineage determination.[18] MiR-21 was also found to be robustly stimulated in RANKL-induced osteoclastogenesis.[71, 97] MiR-223 has been shown to stimulate the expression of M-CSFR; however, overexpression of pre-miR-223 blocks the formation of osteoclasts.[98] Inhibition of miR-223 was noted to down-regulate TRAP-positive osteoclast formation and decrease M-CSFR levels.[71, 73] Zhang et al. found that miR-155, an interferon-β (IFN-β) inducible miRNA, can target suppressor of cytokine signaling 1 (SOCS1) and microphthalmia-associated transcription factor (MITF) and mediate the suppressive effects of IFN-β on osteoclastogenesis.[10] Overexpression of miR-146a has been shown to lead to a significant down-regulation of c-Jun, NFATc1, transcription factor PU.1 and TRAP. The down-regulation of TNF receptor-associated factor 6 (TRAF6), a validated target of miR-146a and critical signaling molecule in RANKL-mediated osteoclastogenesis[111-113] has been found to suppress osteoclastogenesis.[110, 111, 113, 114]

The aim of this study was to investigate the miRNA profiles of monocyte-to-osteoclast differentiation using microarrays over a 12 day period. The secondary aim was to identify differentially expressed miRNAs not previously associated with osteoclast differentiation and function and identify their predicted targets.

Materials and Methods

Isolation of Monocytes and Differentiation into Macrophages and Osteoclasts

Peripheral blood mononuclear cells (PBMCs) were collected from buffy coats derived from four healthy, human, independent donors (Oklahoma Blood Institute, Oklahoma City, OK) by density gradient centrifugation, using Ficoll-paque (Amersham, Uppsala, Sweden.) Monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Auburn, CA) following the manufacturer's instructions and cultured as previously described[115, 116] Briefly, the isolated monocytes are plated in duplicate onto 24-well tissue culture-treated plates (BD Biosciences, San Jose, CA) at a density of 1.2 x 10^6 cells/cm^2 in media consisting of RPMI (Cellgro, Manassas, VA) at 37 C, 5 % CO_2. 10% heat-inactivated fetal bovine serum (FBS, Invitrogen, Carlsbad, CA) and 50µg/mL gentamycin (Sigma, St. Louis, MO) was added after 2 hours.[115, 116] Media was replaced every 3 days throughout the experiment.

For osteoclast differentiation, monocytes were cultured in the presence of a33ng/ML M-CSF and 100ng/ML sRANKL (Peptrotech, Offenbach, Germany) over 12 days. Media was replaced every 3rd day with fresh media containing M-CSF and sRANKL. Freshly isolated monocytes and monocytes cultured in the presence of M-CSF alone were utilized as controls. Day 0, 1, 3, 6 and 12, cultures were used for the study.

Flow Cytometry

For phenotyping of monocytes and macrophages, day 0 monocytes and day 6 macrophages were examined via FACS analysis with regards to the antibodies against $CD14^{FITC}$, $CD86^{FITC}$ and $CDw93^{FITC.}$ Antibodies were purchased from AbD Serotec (Raleigh, NC) and used in the following dilutions and formats: $CD14^{FITC}$ 1/400 dilution, IgG1, mouse α-human; $CD86^{FITC}$ 1/200 dilution, IgG1, mouse α-human and $CDw93^{FITC}$ 1/200 dilution, IgG2bκ, mouse

α-human. Cells were transferred to a 96-well V bottom plate (BD Falcon), kept on ice for 10 minutes and a pipette tip was used to scrape and vigorously pipette up and down. Plates were centrifuged at 1600rpm for 5 minutes and re-suspended in 200µL of FACS buffer (ice-cold PBS, 2mM EDTA, 0.5% FBS.) Centrifugation at 1600rpm was repeated for an additional 5 minutes, then cells were re-suspended in 50µL of FcR block (1/200 dilution, Miltenyi Biotech, α-human.) 50µL of the respective antibodies were diluted 1/100, 50µL of the respective antibody was then added (2x final concentration for 15 minutes.) Centrifugation was repeated at 1600rpm for 5 minutes. Cells were re-suspended in 200µL of FACS buffer, centrifugation repeated at 1600rpm for 5 minutes and cells re-suspended once again in 200µL of 4% paraformaldehyde (PFA) (USB, Affymetrix.) All samples were analyzed by FACS using DellQuest software and data analyzed using FloJo software (TreeStar Inc., Ashland, OR, USA)

IgG is purified and conjugated to Fluorescein Isothiocyanate Isomer 1 (FITC)- liquid. CD14 is a glycoprotein containing multiple leucin-rich repeats, and is found anchored to the cell membrane via glycosylphosphatidylinositol[117] and in a soluble form.[118] CD14 is expressed on the surface of monocytes and macrophage, as well as some non-myeloid cells[119] and serves as a pattern recognition receptor[120, 121] for several ligands, including leukopolysaccharide (LPS.) In coordination with other proteins, CD14 helps to mediate the innate immune response to LPS.[122] CD86 is expressed on antigen presenting cells (APCs) and provides co-stimulatory signals for the activation and survival of T-cells. CD86 has been shown to be expressed on freshly isolated monocytes[123] and serves as a co-stimulatory ligand, along with CD80, for CD28/CTLA-4.[124] CDw93 is used to mark differentiation of monocytes to macrophages as expression ceases throughout the differentiation process and is not expressed on macrophages.[125] Previously known as C1qRp, CD93 has been shown to play a role in cell adhesion.[126]

TRAP Staining

Tartrate resistant acid phosphatase (TRAP) staining was performed on day 0, 3, 6 and 12 using 10mg Naphtol AS-MX phosphate, 1mL dH_2O, 0.2M sodium acetate, 0.2M acetic acid, 0.3M sodium tartrate, 0.1M acetate buffer and TRAP buffer (pH = 5) at 37°C. Following removal of medium and washing with PBS, cells were fixed with 10% glutaraldehyde for 15 minutes at 37°C. Following two subsequent washes with PBS, TRAP stain was added for 5 minutes at 37°C. TRAP stain was removed, wells washed with PBS and cells observed using light microscopy at 20x magnification, to confirm differentiation to osteoclasts.

OsteoLyse™ Assay

The OsteoLyse™ Assay was performed on days 3, 6, and 12. Differentiation was confirmed by microscopy, TRAP + staining and bone resorption using the Osteolyse™ bone resorption kit (Lonza, Walkersville, MD) according to the manufacturer's recommendations. Cells in media containing M-CSF and sRANKL were seeded at a density of $4x10^5$ cells/cm^2 onto the OsteoLyse™ Cell Culture Plate. The macrophages (cultured in the absence of sRANKL) served as a control group for comparison. Cells were cultured for 6 days, then 200µL of Fluorophore releasing Reagent was placed in each well of the included black 96-well assay plate. 10µL of cell culture supernatant was added to these wells and after mixing, fluorescence was measured for each well with excitation of 340nm and emission at 615nm.

Cell Viability

Viability was assayed using CellTiter 96® Aqueous One Solution Reagent (Promega, Madison, WI, USA) following the manufacturer's guidelines. Briefly, cells were plated onto a

96-well plate and 20µL of reagent was added to each well. Following 2 hours of incubation at 37°C, the absorbance was recorded at 490nm using a 96-well plate reader.

Total RNA Isolation

At the indicated collection points, the supernatant was removed and stored separately at -80°C. Total RNA was isolated using the miRNeasy mini kit (Qiagen, Gaithersburg, MD) according to manufacturer's instructions. Isolation was performed on 12 samples simultaneously. Total RNA concentrations and integrity was evaluated using a NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE) and Bioanalyzer (Agilent, Foster City, CA), respectively.

MicroRNA Arrays

RNA samples (N=36) were randomly labeled 1-36 (for blinding purposes) and sent to Exiqon Services (Vedbaek, Denmark) for microarray analysis. RNA quality was confirmed by Exiqon Services. 225ng of RNA were labeled using the miRCURY LNA™ microRNA Hi-Power Labeling Kit Hy3™/HY5™ and subsequently hybridized on the miRCURY LNA™ microRNA Array (7th) MicroRNA levels were probed using miRCURY LNA™ microRNA Arrays following the procedures described by the manufacturer.

Statistical Analysis

Statistical analyses of the microarray data were provided by Exiqon services. *P*-values were corrected for multiple testing by the Benjamini and Hochberg adjustment method. Genes that have been found to be significant via the one-way ANOVA test were subjected to the Tukey's 'Honest Significant Difference' test, thus determining which groups contribute the greatest to the significant difference noted. However, we performed Bonferroni correction upon the original data and found the selection of miRNAs to be more stringent using this format. With

Benjamini and Hochberg adjustment and Tukey's 'Honest Significant Difference,' a subset of 219 miRNAs ($p<0.05$) were identified out of the 422 total miRNAs analyzed. Following the Bonferroni correction using the original data, 69 miRNAs were found to be significantly differentially expressed. Fold change was calculated using the minimum and maximum group averages, indicating that a fold change was calculated from the greatest change in signal throughout the differentiation process, not necessarily from day 0-12, although in some miRNAs, the greatest change occurred from day 0 to day12. Thus, a significant change in expression levels occurred between two time points within the differentiation period.

Network Analysis

Ingenuity® Pathway Analysis was used to examine networks relating the miRNAs which were determined to be differentially expressed during osteoclast differentiation (Bonferroni $p<0.05$). All miRNAs which had calculated Bonferroni $p<0.05$ were input and this analysis was not limited to those with a signal intensity in the range of 7.5-14.5, nor a 2-fold change requirement.

Target Selection

The miRNAs deemed to be significantly differentially expressed, as per Bonferroni correction were further investigated. Many of these miRNAs were also noted in the top 30 (ranked by *p*-value) differentially expressed miRNAs throughout differentiation from monocytes to osteoclasts which were provided from Exiqon Services in their data analysis summary. Prior to target selection, Exiqon Services recommended limiting selection to miRNAs with a fold change of at least 2-fold as well as average signal intensity in the range of 7.5-14.5. *In-silico* analysis was performed using MiRWalk[127] (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) Predicted Targets module to investigate mRNA targets for

these miRNAs. Genes noted to be predicted targets of a significant, differentially expressed human miRNA were investigated for involvement in cell differentiation or specifically osteoclastogenesis and osteoclast function; if the status as a predicted target was agreed upon by a minimum of three of the four following database programs: miRWalk, miRanda (August 2010 release, http://www.microrna.org/microrna.do), miRDB (April 2009 release, http://mirdb.org/miRDB/), and TargetScan Version 5.1 (http://targetscan.org/).

Results

Examination of the flow cytometry data reveals that macrophages had differentiated from peripheral blood monocytes. Figure 1 shows the phenotyping results comparing day 0 monocytes and day 6 monocytes (cultured in M-CSF following monocyte isolation.) Cultures tested positive for CD14, CD86 and CD93. Data represented in red signifies unstained sample, blue coloring represents control for the specific isotype; together these account for background noise and control. Green represents stained sample and the amount of cells positive for the specific cluster of differentiation. Both freshly isolated monocytes and those cultured with M-CSF were CD14+ and CD86+; however, only the monocytes were CD93+. This is consistent with differentiation of the monocytes cultured in M-CSF to macrophages as these latter cells are CD93-. This phenotyping data confirmed the presence of macrophages through successful differentiation.

We next sought to confirm the differentiation of monocytes cultured with M-CSF and sRANKL to osteoclasts. Figure 2 displays the TRAP staining data for day 0 monocytes and those cultured in M-CSF and sRANKL at days 3, 6 and 12. TRAP is a glycosylated monomeric metalloenzyme, which is expressed in mammals,[128] which tends to be localized within the ruffled border, lysosomes, Golgi cisternae and vesicles of the osteoclast.[129] The red stain becomes more

predominant with differentiation to osteoclasts and the multinucleated osteoclasts can be seen under closer higher magnification. Figure 3 shows day 12 osteoclasts at 40x magnification, differentiated from the peripheral blood monocytes, clearly noting multiple nuclei and the fingerlike processes that make up the ruffled border, the structure that facilitates delivery of enzymes and thus creates an acidic environment for bone degradation.

The OsteoLyse™ Assays were performed on days 3, 6 and 12 for cells cultured for the macrophage lineage (M-CSF) as well as the osteoclast lineage (M-CSF +sRANKL.) This assay quantitatively measures *in vitro* osteoclast-mediated bone resorption by directly measuring the release of matrix metalloproteinases into the resorption lacuna of the osteoclast.[130] The release of Eu-labeled collagen fragments was measured after the cell culture supernatant was added to Fluorophore-Releasing Reagent and counted using time-resolved fluorescence.[131] As can be noted in Figure 4, these results demonstrate very minimal differences at early time points between macrophage and osteoclasts, prior to day 12. However, the large, multinucleated, TRAP+ cells noted at day 12 indicate osteoclast differentiation was progressing normally, as their morphology was clearly different compared to macrophage cultures (Figure 3.)

RNA samples were labeled using the miRCURY LNA™ microRNA Hi-Power Labeling Kit Hy3™/Hy5™ and hybridized on the miRCURY LNA™ microRNA Array (7^{th}.) MiRNA levels were then interrogated using miRCURY LNA™ microRNA Arrays. The manufacturer reports that the Hi-Power Labeling Kit allows highly efficient and uniform labeling, the hybridization and washing steps are fully automated, allowing for excellent reproducibility and arrays are scanned in an ozone-free environment to minimize day to day variation. *P*-values and Bonferroni correction were calculated for maximum change of expression for each miRNA. This was not necessary the overall change, from day 0 to 12, as the maximum change may have

occurred between two different time points. Table 1 lists the 69 miRNAs that were differentially expressed throughout osteoclast differentiation as per Bonferroni correction (p<0.5). Using these 69 miRNAs, ANCOVA p-values were then calculated for the change in expression value for each day compared to the baseline, day 0. Figure 5 shows that from day 1 to day 12, the number of miRNAs differentially expressed as compared to baseline increases from 58 on day1 to 64 on day 3, 67 on day 6 and 69 miRNAs on day 12. Further analysis of target mRNAs was limited to those with an expression level (average Hy3) within a range of 7.5-14.5 as well as a fold change of at least 2 (log fold change $\geq$1.0, Exicon.) Table 2 shows the 27 remaining miRNAs from the original list of 69 which were stated to be differentially expressed based on Bonferroni correction alone. Table 2 lists the miRNAs in order of log fold change, with those at the top being those that were up-regulated with the greatest log fold change and those at the bottom being those that were down-regulated with the greatest log fold change. The negative value indicates down-regulation of the particular miRNA. 15 of 27 miRNAs were up-regulated throughout the differentiation of osteoclasts. These include miR-4732-5p, miR-146a-5p, miR-3940, miR-4497, miR-146b-5p, miR-193a-3p, miR-378a-3p, miR-378d, miR-1246, miR-4456, miR-3182, miR-29a-3p, miR-22-3p, miR-378c and miR-3676-5p. 12 of the 27 miRNAs were down-regulated throughout osteoclastogenesis and include miR-101-3p, miR-103a-3p, miR-191-5p, miR-142-5p, miR-30c-5p, miR-142-3p, miR-92a-3p, miR-15a-5p, miR-15b-5p, miR-16-5p, miR-106b-5p and miR-223-3p. Figures 6a-f display the signal or Hy3 expression from day 0-12 for six of these miRNAs; miR-146b-5p, miR-146a-5p, miR-29a-3p and miR-1246 which have been up-regulated, and miR-223-3p and miR-106b-5p which have been down-regulated. While the expression levels of miR-1246 decrease after an initial increase from day 0 to 3, the overall change is an increase and so this miRNA is listed as up-regulated. Values for all four donors

have been entered at each time point and the error bars show mi deviation at each time point. For a given miRNA, the patterns in expression level change have been fairly consistent between the donors.

The top 69 differentially expressed miRNAs, as per Bonferroni correction only, were entered into Ingenuity® Pathway Analysis. Figure 7 shows the associated network functions in relation to cancer, gastrointestinal disease, cellular assembly and organization. MiRNAs miR-455-5p, miR-455, miR-22-3p, miR-210-3p, miR-29, mir-29b-3p, miR-1237-5p, miR-378 and miR-378a-3p are shown in this network along with the relationships with the other molecules in the network. Table 3 lists the genes and their function. The majority of these listed functions relate cellular proliferation, differentiation and growth, and in some cases, apoptosis.

Target mRNAs were investigated from 27 differentially expressed miRNAs, as per *p*-value with Bonferroni correction, and limiting for those with expression value within range of 7.5-14.5 and fold change $\geq$2. Targets were initially identified with the miRWALK database, allowing for comparison with 3 other publically available databases. Table 4 includes a list of these 27 top differentially expressed miRNAs and target mRNAs noted in miRWALK with a brief gene description. Only those mRNAs that were noted to have functions or roles in osteoclastogenesis, and were predicted targets in at least 3 of the 4 databases were included. When a search did not yield information on targets for a given miRNA on miRWALK, the miRDB database was used; these mRNAs are marked with an asterisk, as miRDB does not examine agreement between multiple programs as miRWALK does. Each of the top 30 mRNA targets was investigated for each miRNA, whether in miRWALK or miRDB. After this, a manual search was conducted for particular mRNAs known to be associated with osteoclastogenesis. Several of the miRNAs in this table have "unknown" listed under the mRNA

column, including miR-3940-5p, miR-4497, miR-378d, miR-378c, miR-3676-5p and miR-191-5p. For these miRNAs, the investigation did not yield any predicted mRNA targets that were associated with osteoclastogenesis. Several of the mRNA targets are listed as predicted targets for multiple miRNAs. For example, FOSL2 is targeted by both miR-193a-3p and miR-92a-3p, and the members of the FOS gene family have been implicated in regulatory roles of cell proliferation and differentiation. These are not the only miRNAs that target FOSL2; the miRDB database indicates that FOSL2 is predicted to target 34 different miRNAs. FOS and FOSB are also members of this same gene family and are targeted by miR-101-3p and miR-142-5p, respectively. TGFBR1, which is predicted to be targeted by 151 miRNAs in miRDB, was targeted by 6 of our top 27 miRNAs, including miR-193a-3p, miR-3182, miR-22-3p, miR-101-3p, miR-142-5p and miR-142-3p. ITGB3 was predicted to be targeted by miRNAs in miRDB, and was targeted by 4 of our top 27 miRNAs, including miR-146b-5p, miR-193a-3p, miR-1246 and miR-92a-3p. Both of these genes were proposed to have key roles in RANKL and M-CSF induced differentiation of osteoclasts as they interacted with many proteins and were noted to be key nodes in signaling transduction.[132]

Figures 8 and 9 display the significantly differentially expressed miRNAs that have been up-regulated and down-regulated, respectively. The least square means were calculated using the expression values for each donor at every time point for these miRNA. Among the donors, the expression values for a given miRNA at each time point were closely related (data not shown.) Both figures display that the levels of expression (average Hy3) at each time point vary for the different miRNAs, and for a given miRNA, the expression values vary at different time points. Note that the peak or minimum expression values do not always occur at day 12.

Discussion

The present study examines the miRNA profile and predicted mRNA targets throughout the process of osteoclastogenesis. Peripheral blood monocytes were isolated from 4 independent, healthy, human donors and cultured in media containing M-CSF and sRANKL to induce osteoclastogenesis. Day 0 monocytes and macrophages cultured with M-CSF and no sRANKL were used as for controls for comparison, particularly in confirmation of differentiation to osteoclasts. Flow cytometry was used to phenotype monocytes and macrophages, confirming the differentiation to macrophages by comparing day 0 to day 6 samples. At day 6, samples were both CD14 and CD86+ but negative for CDw93, although the day 0 samples were CDw93+. These findings were typical of differentiation to macrophages as CDw93 expression is typically lost in monocyte-to-macrophage differentiation. TRAP staining and the Osteolyse™ assay were then used to confirm differentiation to osteoclasts in the appropriate samples. In comparison of TRAP stain for samples at days 0, 3, 6 and 12; TRAP positivity increases with expected osteoclast differentiation. The Osteolyse™ assay was performed on days 3, 6 and 12 on both samples cultured for macrophage differentiation and those cultured for osteoclast differentiation. Interestingly, there was little to no difference up to day 12, between macrophages and osteoclasts. This pattern was consistent in the Osteolyse™ data for all 4 donors. Cody et al. 2011 proposed a different method for generation of osteoclasts *in vitro*, by differentiating osteoclasts from bone marrow cells. However, in this study, although they confirmed TRAP+ staining at an earlier time point, they confirmed the presence of resorption pits using scanning electron microscopy, upon the devitalized bovine cortical bone their osteoclast precursors were seeded upon. Cody et al. maintained cultures in osteoclastogenic media for 18 days before examining for resorption pits to confirm function.[133] One possible

explanation for the similarity in Osteolyse™ data between our samples may be the time point at which we conducted the assay, which was a maximum of 12 days. However, the large, multinucleated, TRAP+ cells noted at day 12 clearly indicate osteoclast differentiation was progressing normally (Figure 3.)

Following total RNA isolation, samples were randomly labeled 1-36 to minimize bias during the labeling of samples and hybridization onto the arrays. Quality control measures were repeated by Exiqon to confirm quantity and integrity of the RNA. In addition, Exiqon services examined the number of miRNAs above background threshold. Numbers were found to be within the expected range and were found to be comparable for all samples, indicating comparable quality of samples.

Microarray technology has been used successfully in the past in genomic and biologic research[71, 134] and has been found to enhance binding affinity as well as improve specificity and sensitivity of miRNA detection.[71, 135] To account for high throughput experimental procedures, such as microarrays, being prone to giving false positive results, Exiqon Services provided p-values which have been corrected for multiple testing by Benjamini and Hochberg adjustment method. Controlling for the number of false positives when a large number of statistical tests are completed simultaneously helps to prevent type I error, where the null hypothesis is incorrectly rejected.[136] MiRNAs which were found to be significantly differentially expressed by one-way ANOVA test were subjected to Tukey's 'Honest Significant Difference' test in order to determine which groups contributed the most to the significant difference. With the analysis, throughout monocyte to osteoclast differentiation, 403 of 422 miRNAs analyzed were found to be up or down regulated, and a subset of 219 miRNAs were shown to be significantly differentially expressed ($p < 0.05$). To increase stringency, we calculated *p*-values and performed

a Bonferroni correction. 69 miRNAs were found to be significantly, differentially expressed (p <0.05) between two time points within the 12 days. MiRNAs with expression levels (Hy3) within a range of 7.5-14.5 and with a minimum 2-fold change were further selected before analyzing for target genes (Exiqon.) 27 miRNAs formed the final list of differentially expressed miRNAs throughout osteoclastogenesis. It is recognized that a less stringent selection criteria would have revealed more miRNAs and thus targets could be investigated. For example, miR-210, which was listed in our 69 differentially expressed miRNA but not selected in our final 27, has been suggested to be involved in TNFα-regulated osteoclast differentiation.[137] Future studies with greater numbers of replicates are required for further evaluation.

Target selection was completed using the publically available mirWALK database, which allowed for comparison between multiple similar databases. The three that were chosen for comparison with mirWALK were TargetScan, miRANDA and miRDB. Such programs predict miRNA targets by identifying conserved complementary motifs in the 3' UTR of target mRNAs and the seed sequence, or 5' proximal end of a mature mRNA.[71, 138] TargetScan[139] and miRANDA[140] are among the most commonly used target prediction programs.[71] In 2006, Sethupathy et al. compared the performance of different computational methods, studying 84 miRNA-target gene interactions, involving a total of 32 miRNAs. The three algorithms investigated (PiCTar, TargetScan and miRANDA), identified approximately 65% of conserved, unbiased interactions, which were supported by experiments.[77, 141] Although the different programs do have their individual false positive rates, the comparison of the predictions from the programs can lead to enhancement of the specificity for prediction of targets. The combination of the programs could predict approximately 92% of the conserved and verified interactions between miRNAs and the target genes.[142] Although this method is able to achieve high

probability predictions, as algorithms are combined, false negatives may become problematic, highlighting that the selection of algorithms is critical.[142] Thus far, the *in silico* approach to identifying miRNA targets has been the main method, and it does allow for the identification of biological roles of miRNAs.[77]

Our search through mirWALK and miRDB for target mRNAs did not yield any related to osteoclastogenesis for some of the miRNAs. Although such predicted targets could not be determined in the present study, this does not mean they do not exist, and future research may ascertain osteoclast-related targets of these miRNAs. As can be noted in Table 4, miRNAs target multiple mRNAs, and a given mRNA may be a target for multiple miRNAs. This results from the partial complementarity with which miRNAs bind to their targets, and as this is over a short sequence, therein lays a challenge to the identification of target genes.[143]

This study has some limitations. Although we used biological replicates (4 independent donors) and evaluated miRNA expression over 12 days (N=36 arrays), a greater number of replicates and a longer differentiation period (ex. up to 20 days) would likely reveal additional miRNAs important in the differentiation process. RT-PCR validation will be required to confirm our array findings, in addition to functional assays (overexpression/knockdown) to confirm the *in-silico* findings. Furthermore, as our Osteolyse™ data illustrates, the differentiation process in the present study yielded pre-functional osteoclasts.

In conclusion, 27 miRNAs were differentially expressed throughout 12 day of osteoclastogenesis, culturing of peripheral blood monocytes in media containing M-CSF and sRANKL. Several predicted targets associated with osteoclastogenesis were identified for these miRNA. Further, our bioinformatics analysis revealed that many of these miRNA targeted

multiple mRNAs and that individual mRNAs were also targets for multiple miRNA. It has previously been reported that miRNAs, such as miR-223 and miR-146a are associated with osteoclastogenesis[98,110] To our knowledge, miR-378a and miR-3182, identified in this study, have not been reported to be associated with osteoclastogenesis in the literature. The miRNAs investigated in this study may play a crucial role in osteoclastogenesis and thus bone resorptive disease, providing a basis for further investigations into mRNA targets, disease pathogenesis and possibly, future therapeutic targets.

FIGURE 1

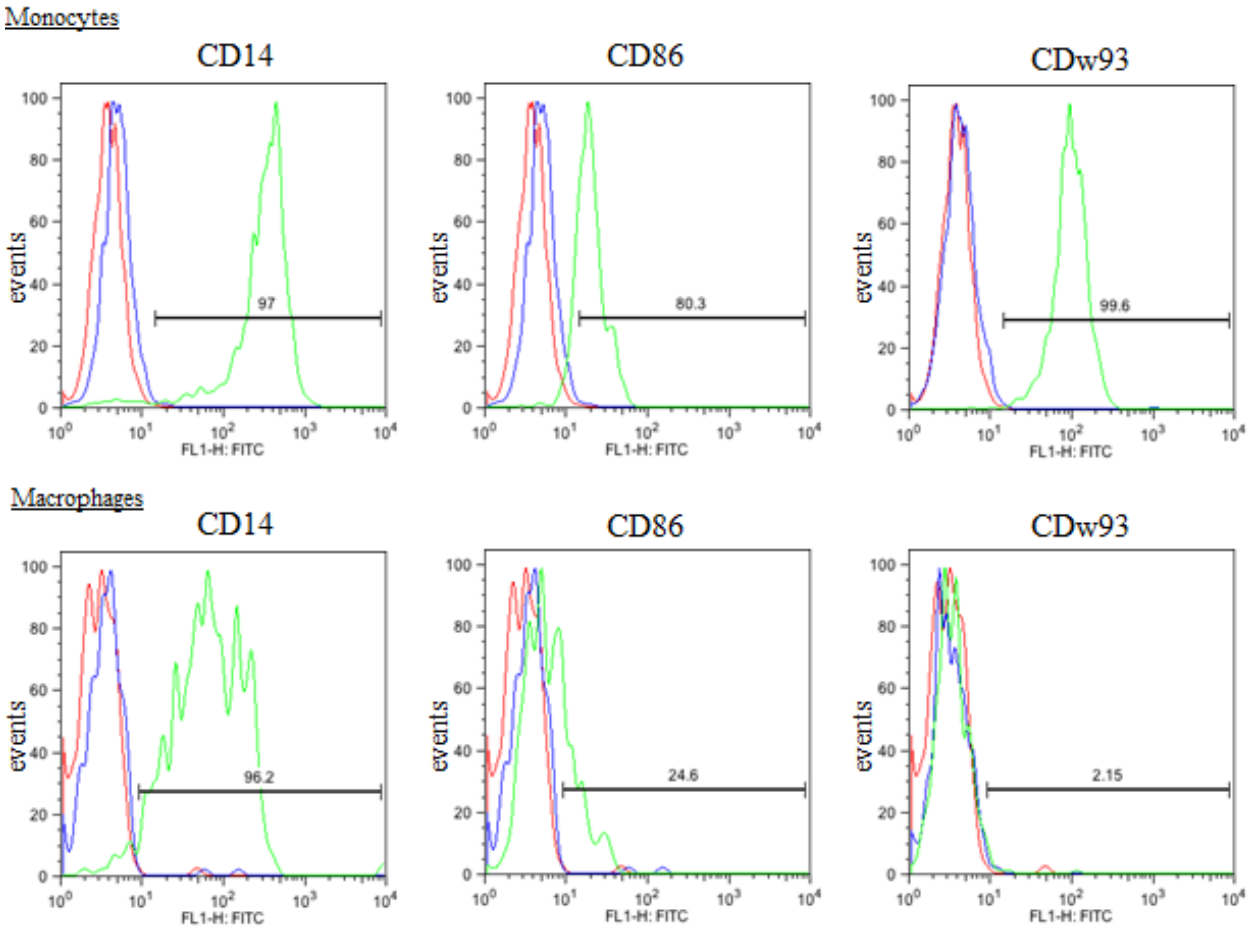

Figure 1: Phenotyping of day 0 monocytes and day 6 cells cultured with M-CSF using Flow Cytometry with FITC to confirm differentiation to macrophages. Red- unstained, blue- isotype control, green- sample. Both samples are CD14 and CD86 positive. Only the monocytes are CD93+ which is consistent with differentiation to macrophages in the day 6 samples cultured with M-CSF.

FIGURE 2

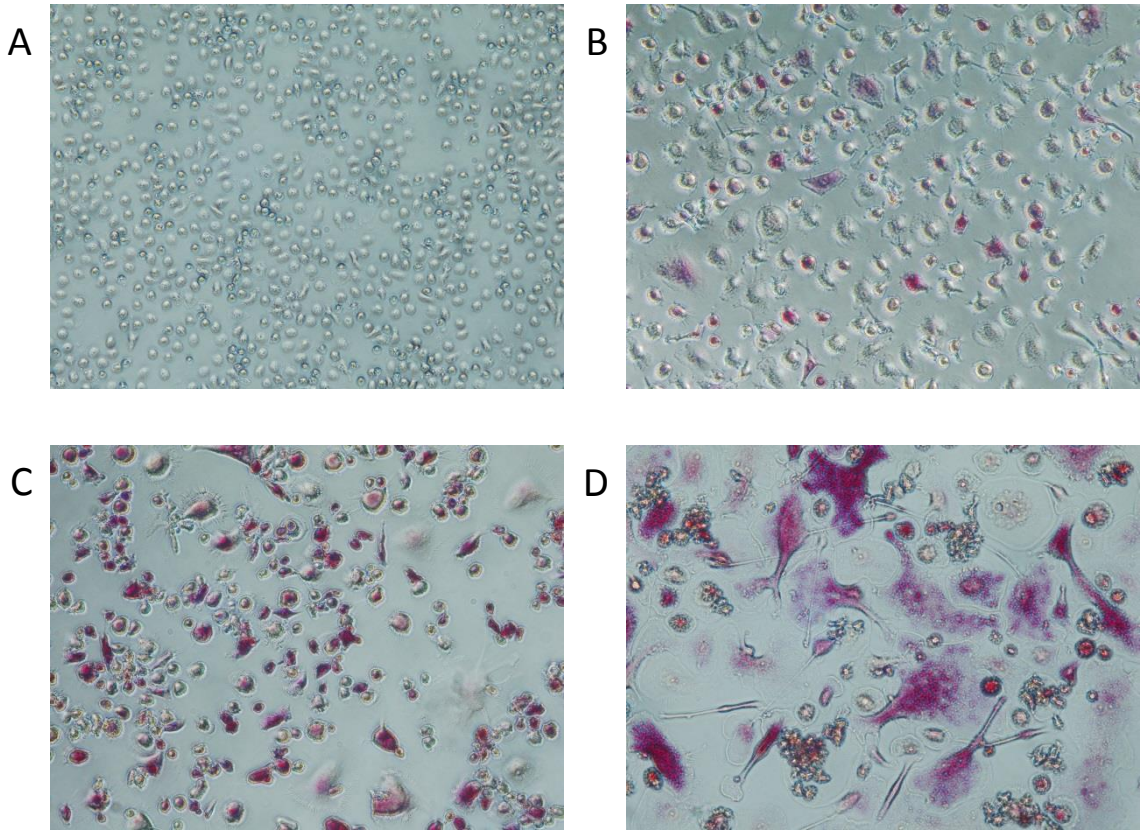

Figure 2: Representative TRAP stain of differentiating osteoclasts (20x magnification.) A) Day 0 monocytes B) Day 3 C) Day 6 D) Day 12 noting progressively larger cells and gain in staining intensity. Note multinucleated morphology of day 12 cultures.

FIGURE 3

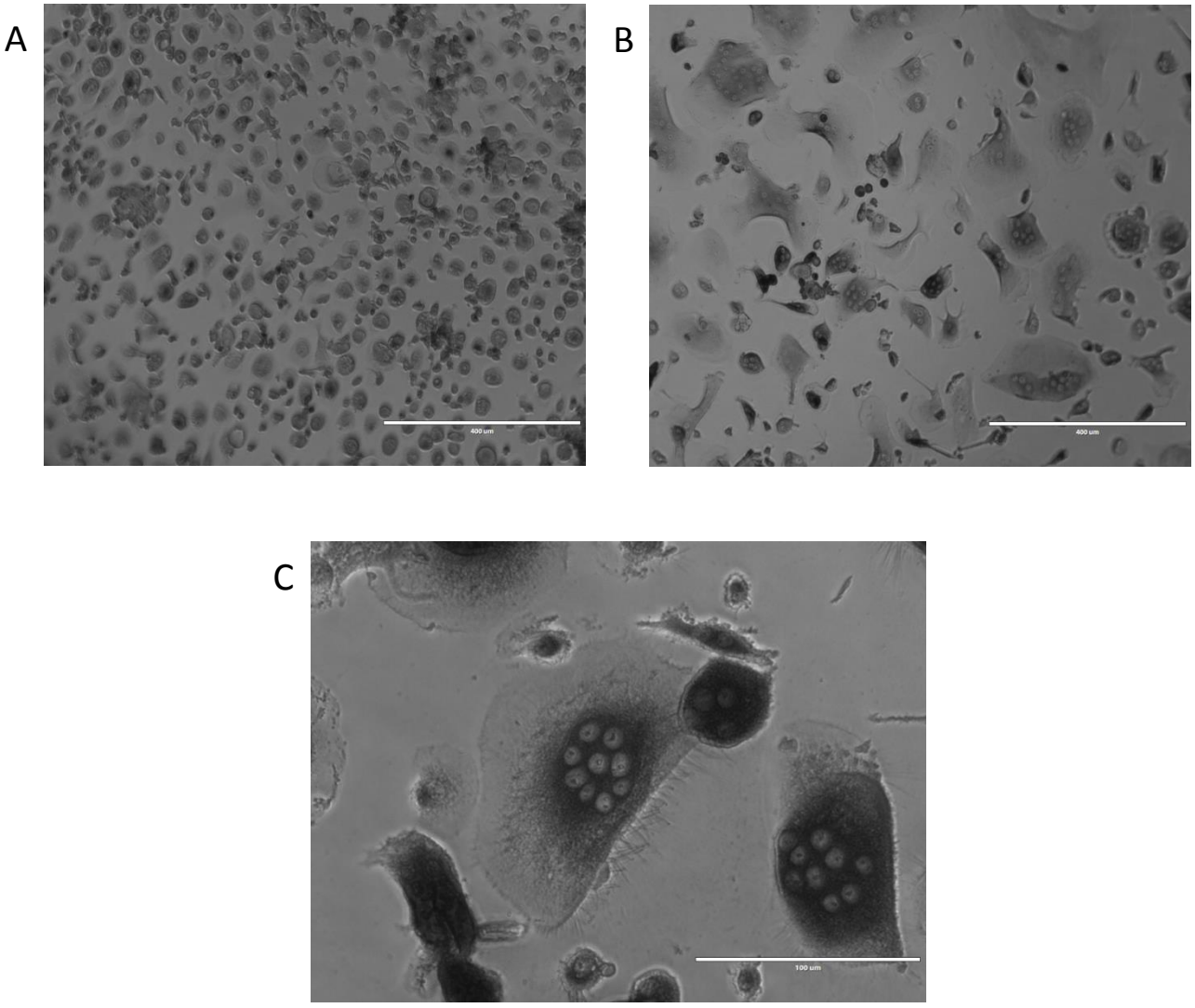

Figure 3: Representative cells at two time points. A) Day 0 monocyte B) Day 12 osteoclast, note multinucleated cells C) Day 12 osteoclast note large, multinucleated cell with ruffled borders.

FIGURE 4

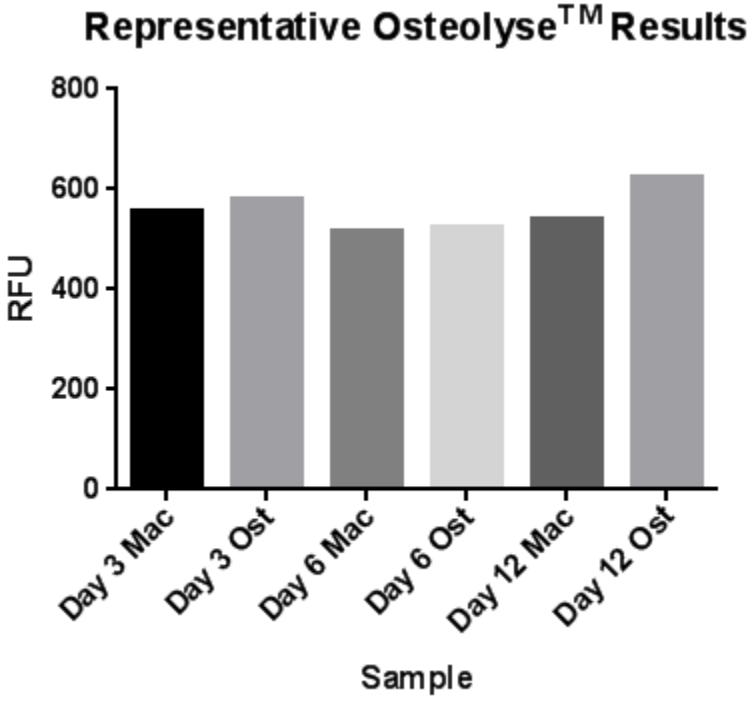

Figure 4: Representative Osteolyse™ results; performed on days 3, 6 and 12 on macrophage and osteoclast lineage samples. Note little to no difference between time points and between macrophages and osteoclasts up to day 12. Pattern of results was found to be consistent for all four donors.

TABLE 1

miRNA	*p*-value Bonferroni	miRNA	p-value Bonferroni
hsa-miR-125a-5p	<.0001	hsa-miR-30c-5p	0.0015
hsa-miR-223-3p	<.0001	hsa-miR-191-5p	0.0017
hsa-miR-106b-5p	<.0001	hsa-miR-940	0.0023
hsa-miR-146b-5p	<.0001	hsa-miR-642b-5p	0.0027
hsa-miR-92a-3p	<.0001	hsa-miR-378f	0.0028
hsa-miR-581	<.0001	hsa-miR-199b-5p	0.0031
hsa-miR-103a-3p	<.0001	hsa-miR-30e-3p	0.0035
hsa-miR-25-3p	<.0001	hsa-miR-455-5p	0.0039
hsa-miR-378a-3p	<.0001	hsa-miR-142-5p	0.0047
hsa-miR-301a-3p	<.0001	hsa-miR-18b-5p	0.0053
hsa-miR-15a-5p	<.0001	hsa-miR-22-3p	0.0054
hsa-miR-193a-3p	<.0001	hsa-miR-3940-5p	0.0055
hsa-miR-378c	<.0001	hsa-miR-130a-3p	0.0058
hsa-miR-454-3p	<.0001	hsa-miR-4497	0.0066
hsa-miR-34a-5p	<.0001	hsa-miR-3178	0.0068
hsa-miR-378d	<.0001	hsa-miR-101-3p	0.0073
hsa-miR-146a-5p	<.0001	hsa-miR-3613-5p	0.0094
hsa-miR-99b-3p	<.0001	hsa-miR-20b-5p	0.011
hsa-miR-339-5p	<.0001	hsa-miR-340-5p	0.0137
hsa-miR-193a-5p	<.0001	hsa-miR-26a-5p	0.0151
hsa-miR-140-3p	0.0001	hsa-miR-365a-3p/hsa-miR-365b-3p	0.0187
hsa-miR-3182	0.0001	hsa-miR-107	0.0187
hsa-miR-29a-3p	0.0001	hsa-miR-660-3p	0.0192
hsa-miR-425-5p	0.0003	hsa-miR-33a-5p	0.0206
hsa-miR-363-3p	0.0003	hsa-miR-4521	0.0223
hsa-miR-15b-5p	0.0003	hsa-miR-423-3p	0.0226
hsa-miR-4732-5p	0.0004	hsa-miR-4780	0.0238
hsa-miR-17-3p	0.0005	hsa-miR-378e	0.026
hsa-miR-130b-3p	0.0007	hsa-miR-4488	0.0262
hsa-miR-142-3p	0.001	hsa-miR-3676-5p	0.0318
hsa-miR-210	0.0011	hsa-miR-16-5p	0.0322
hsa-miR-1246	0.0011	hsa-miR-338-3p	0.036
hsa-miR-500a-5p	0.0011	hsa-miR-197-3p	0.0469
hsa-miR-4456	0.0011	hsa-miR-532-3p	0.0498
hsa-miR-9-5p	0.0012		

Table 1: 69 miRNAs found to be significantly differentially expressed throughout osteoclast differentiation. Benjamini and Hochberg adjustment applied (p <0.05).

FIGURE 5

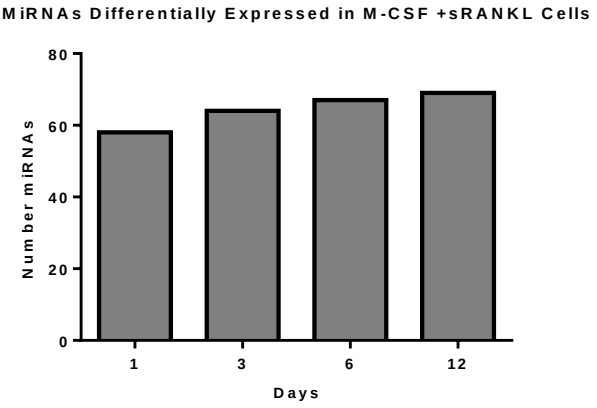

Figure 5: Number of miRNAs differentially expressed in M-CSF + sRANKL cells at each time point (Bonferroni p <0.05) for expression for expression change of miRNA within 12 days and ANCOVA (p <0.05) at specific time point compared to baseline (day 0)

TABLE 2

miRNA	logFc	*p*-value (Bonferroni)	Avg. Hy3
hsa-miR-4732-5p	4.183	0.0004	7.529
hsa-miR-146a-5p	3.274	<.0001	8.034
hsa-miR-3940-5p	3.038	0.0055	9.132
hsa-miR-4497	2.82	0.0066	7.589
hsa-miR-146b-5p	2.408	<.0001	8.226
hsa-miR-193a-3p	2.385	<.0001	8.324
hsa-miR-378a-3p	2.106	<.0001	8.907
hsa-miR-378d	2.065	<.0001	7.915
hsa-miR-1246	1.78	0.0011	11.648
hsa-miR-4456	1.663	0.0011	9.321
hsa-miR-3182	1.658	0.0001	10.544
hsa-miR-29a-3p	1.59	0.0001	8.482
hsa-miR-22-3p	1.398	0.0054	10.161
hsa-miR-378c	1.339	<.0001	7.924
hsa-miR-3676-5p	1.011	0.0318	8.690
hsa-miR-101-3p	-1.252	0.0073	7.879
hsa-miR-103a-3p	-1.273	<.0001	8.975
hsa-miR-191-5p	-1.312	0.0017	8.589
hsa-miR-142-5p	-1.312	0.0047	9.353
hsa-miR-30c-5p	-1.335	0.0015	8.222
hsa-miR-142-3p	-1.57	0.001	11.154
hsa-miR-92a-3p	-1.618	<.0001	7.807
hsa-miR-15a-5p	-2.001	<.0001	9.515
hsa-miR-15b-5p	-2.34	0.0003	8.217
hsa-miR-16-5p	-2.353	0.0322	9.808
hsa-miR-106b-5p	-2.708	<.0001	8.261
hsa-miR-223-3p	-3.744	<.0001	10.224

Table 2: 27 miRNAs found to be significantly differentially expressed throughout osteoclast differentiation. Bonferroni correction applied to p-values. MiRNAs listed by logFc with positive values representing up-regulation and negative values representing down-regulation. *P*-values and expression intensity (average Hy3) also displayed as selection for a fold change $\geq$2 and expression value within 7.5-14.5 range completed.

FIGURE 6

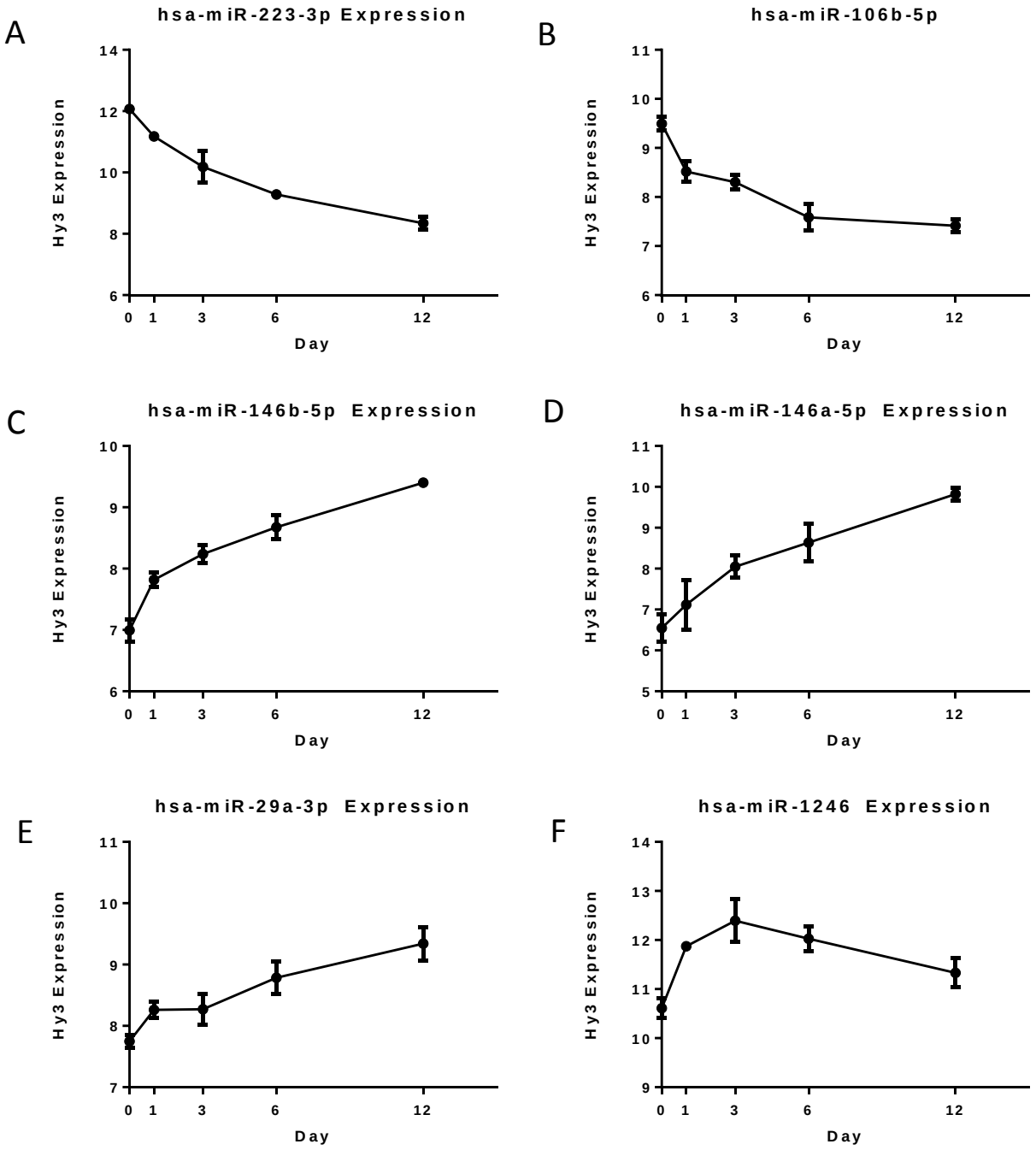

Figure 6: Signal intensity or Hy3 expression at day 0, 1, 3, 6 and 12 for six significantly differentially expressed miRNAs. Error bars display standard deviation at each time point. Levels of expression value are fairly consistent between the 4 donors

FIGURE 7

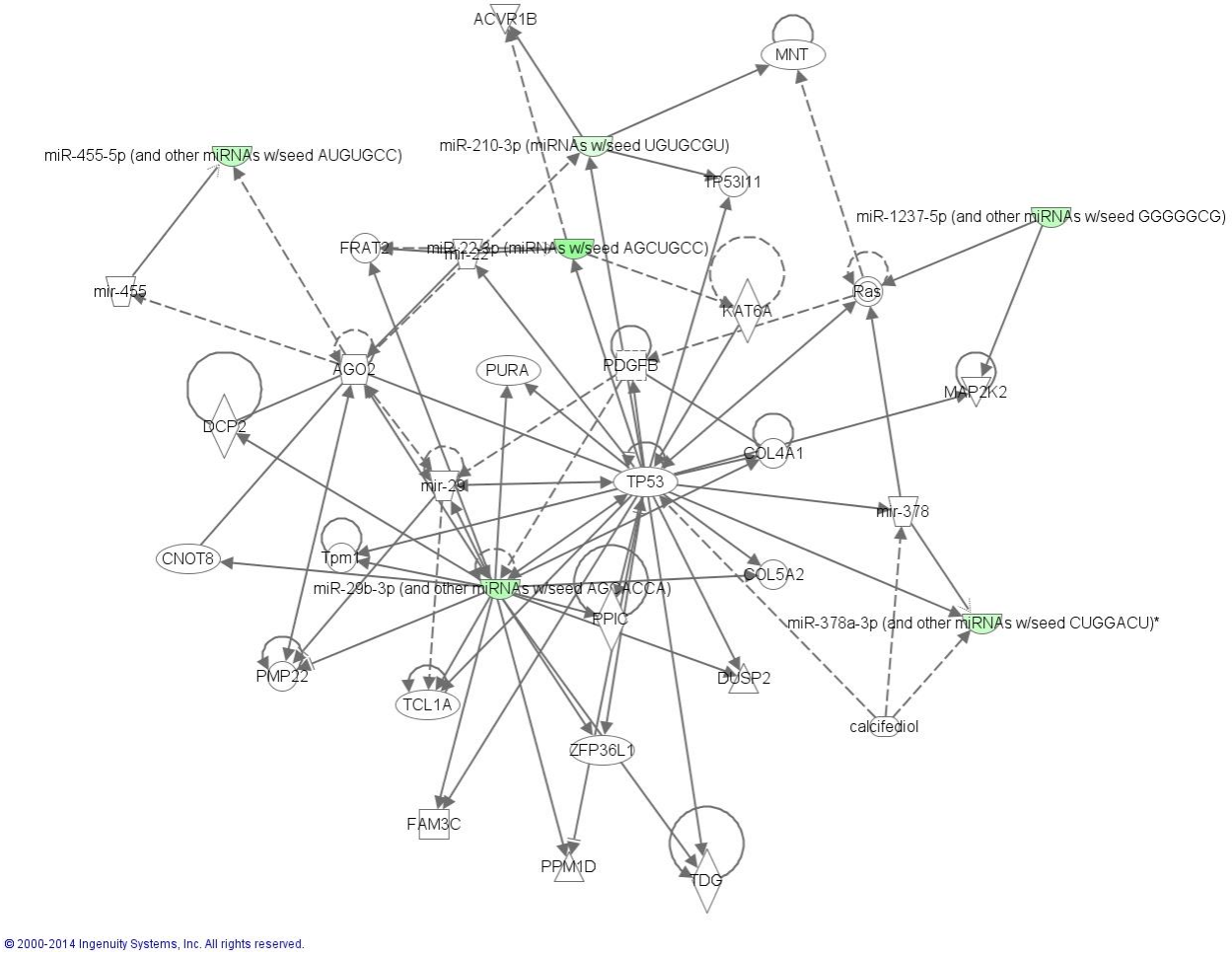

Figure 7: 27 Network with associated functions in cancer, gastrointestinal disease, cellular assembly and organization as determined by Ingenuity® Pathway Analysis, following input of differentially expressed miRNAs during osteoclast differentiation (Bonferroni $p < 0.05$). Green shading indicated down-regulation of miRNA

TABLE 3

Gene	Full name	Role in Cell
MNT	MAX dimerization protein	proliferation, apoptosis, cell cycle progression, function, aging, survival, transformation, expansion, quantity, development
ACVR1B	Activin A receptor, type IB	differentiation, apoptosis, G1/S phase transition, growth
TP53I11	Tumor protein p53 inducible protein 11	proliferation, lysis
RAS	Rat sarcoma	transformation, proliferation, growth, differentiation, apoptosis, activation in, colony formation, senescence, migration, cell cycle progression
MAP2K2	Mitogen-activated protein kinase kinase 2	cell death, transformation, migration, M phase, cell cycle progression, cell spreading, growth, apoptosis, proliferation, replication
COL4A1	Collagen, type IV, alpha 1	formation, abnormal morphology, migration, phosphorylation in, anoikis, development, structural integrity, transdifferentiation, transmigration, proliferation
Calcifediol		expression in, apoptosis, cell death, function, development, action in, differentiation, proliferation
KAT6A	K(lysine) acetyltransferase 6A	number, abnormal morphology, differentiation, lack expression in several diseases
COL5A2	Collagen, type V, alpha 2	organization, abnormal morphology
DUSP2	Dual specificity phosphatase 2	pluripotency, activation in, function, tumorigenicity, apoptosis

PDGFB	Platelet-derived growth factor beta polypeptide	proliferation, migration, transformation, expression in, abnormal morphology, chemotaxis, growth, quantity, activation, recruitment
TP53I11	Tumor protein p53	apoptosis, cell cycle progression, proliferation, cell death, growth, expression in, G1 phase, senescence, transformation, survival
TDG	Thymine-DNA glycosylase	demeythylation in bleeding, edema and several disease conditions
PPIC	Peptidylprolyl isomerase C (cyclophilin C)	invasion, migration, replication
ZFP36L1	ZFP36 ring finger protein-like 1	proliferation, translation, apoptosis
PPM1D	Protein phosphatase, Mg2+/Mn2+ dependent, 1D	apoptosis, proliferation, dephosphorylation in, quantity, cell death, phosphorylation in, G2/M phase transition, mitosis, polyploidization, expression in several disease conditions/cancers
PURA	Purine-rich element binding protein A	proliferation, number, abnormal morphology, quantity, translation in, formation, G2 phase, mitosis, G1 phase, size
FAM3C	Family with sequence similarity 3, member C	
FRAT2	Frequently rearranged in advanced T-cell lymphomas 2	
AGO2	Argonaute RISC catalytic component 2	expression in, binding in, growth, proliferation, processing in, accumulation in, quantity, assembly in, maturation in, invasiveness
TCL1A	T-cell leukemia/lymphoma 1A	apoptosis, proliferation, expression in, survival, methylation in, maintenance, function in, growth, activation in, activation
Tpm1	Tropomyosin 1, alpha	actin capping, shape, size, migration, activity, maximum tension, contractility, formation, quantity

PMP22	Peripheral myelin protein 22	abnormal morphology, myelination, morphology, differentiation, proliferation, assembly, action potential, synaptic transmission, migration, cell death
DCP2	Decapping mRNA1	degradation
CNOT8	CCR4-NOT transcription complex, subunit 8	proliferation, repression in, translation

Table 3: Targets noted in Figure 7 along with name and role played within cell. Note common functions of cell proliferation, differentiation, morphology and apoptosis

TABLE 4

miRNA	*mRNA*	*Gene description*
hsa-miR-4732-5p	*RBPJ**	*recombination signal binding protein for immunoglobulin kappa J region*
hsa-miR-146a-5p	*TRAF6**	*TNF receptor-associated factor 6, E3 ubiquitin protein ligase*
hsa-miR-3940-5p	*unknown*	*---*
hsa-miR-4497	*unknown*	*---*
hsa-miR-146b-5p	*ARF6*	*ADP-ribosylation factor 6*
hsa-miR-146b-5p	*ITGβ3*	*integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)*
hsa-miR-146b-5p	*RAC2*	*ras-related C3 botulinum toxin substrate 2*
hsa-miR-146b-5p	*IL17A*	*interleukin 17A*
hsa-miR-193a-3p	*ITGβ3*	*integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)*
hsa-miR-193a-3p	*FOSL2*	*FOS-like antigen 2*
hsa-miR-193a-3p	*TGFβR1*	*transforming growth factor, beta receptor I*
hsa-miR-378a-3p	*ELK4**	*ETS-domain protein (SRF accessory protein 1)*
hsa-miR-378d	*unknown*	*---*
hsa-miR-1246	*CTSK*	*cathepsin K*
hsa-miR-1246	*CA2*	*carbonic anhydrase II*
hsa-miR-1246	*ANK1*	*ankyrin 1, erythrocytic*
hsa-miR-1246	*ITGβ3*	*integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)*
hsa-miR-4456	*ERO1Lβ**	*ERO1-like beta*
hsa-miR-3182	*TGFβR1**	*transforming growth factor, beta receptor I*
hsa-miR-29a-3p	*BIRC2*	*baculoviral IAP repeat containing 2*
hsa-miR-29a-3p	*GSR*	*glutathione reductase*
hsa-miR-29a-3p	*CA2*	*carbonic anhydrase II*
hsa-miR-29a-3p	*MEFV*	*Mediterranean fever*
hsa-miR-22-3p	*TGFβR1*	*transforming growth factor, beta receptor I*
hsa-miR-22-3p	*CIC*	*capicua transcriptional repressor*
hsa-miR-378c	*unknown*	*---*
hsa-miR-3676-5p	*unknown*	*---*
hsa-miR-101-3p	*TGFβR1**	*transforming growth factor, beta receptor I*
hsa-miR-101-3p	*MITF**	*microphthalmia-associated transcription factor*
hsa-miR-101-3p	*FOS**	*FBJ murine osteosarcoma viral oncogene homolog*
hsa-miR-103a-3p	*CA2**	*carbonic anhydrase II*
hsa-miR-103a-3p	*ANK1**	*ankyrin 1, erythrocytic*
hsa-miR-191-5p	*unknown*	*---*

hsa-miR-142-5p	*CIC*	*capicua transcriptional repressor*
hsa-miR-142-5p	*TGFβR1*	*transforming growth factor, beta receptor I*
hsa-miR-142-5p	*CREB1*	*cAMP responsive element binding protein 1*
hsa-miR-142-5p	*FOSB*	*FBJ murine osteosarcoma viral oncogene homolog*
hsa-miR-30c-5p	*CREB1**	*cAMP responsive element binding protein 1*
hsa-miR-142-3p	*TGFβR1*	*transforming growth factor, beta receptor I*
hsa-miR-92a-3p	*DUSP10*	*dual specificity phosphatase 10*
hsa-miR-92a-3p	*CIC*	*capicua transcriptional repressor*
hsa-miR-92a-3p	*MEFV*	*Mediterranean fever*
hsa-miR-92a-3p	*ITGβ3*	*integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)*
hsa-miR-92a-3p	*FOSL2*	*FOS-like antigen 2*
hsa-miR-92a-3p	*MITF*	*microphthalmia-associated transcription factor*
hsa-miR-15a-5p	*CA2**	*carbonic anhydrase II*
hsa-miR-15a-5p	*STXBP3**	*syntaxin binding protein 3*
hsa-miR-15b-5p	*CA2**	*carbonic anhydrase II*
hsa-miR-15b-5p	*STXBP3**	*syntaxin binding protein 3*
hsa-miR-16-5p	*CA2**	*carbonic anhydrase II*
hsa-miR-16-5p	*STXBP3**	*syntaxin binding protein 3*
hsa-miR-106b-5p	*MAP3K2**	*mitogen-activated protein kinase kinase 2*
hsa-miR-106b-5p	*CTSK**	*cathepsin K*
hsa-miR-223-3p	*KHDRBS1*	*KH domain containing, RNA binding, signal transduction associated 1*
hsa-miR-223-3p	*ERP29*	*endoplasmic reticulum protein 29*
hsa-miR-223-3p	*TRAP1*	*TNF receptor-associated protein 1*

Table 4: 27 miRNAs differentially expressed (Bonferroni p <0.05) with signal expression levels between 7.5-14.5 and minimum 2-fold change. One mRNA target associated with osteoclastogenesis is listed for each miRNA. An asterisk next to target indicates that it was ascertained through miRDB database (predicted by MirTarget2), which was done if results were not yielded through miRWALK database. "Unknown" is noted under mRNA targets for tor those miRNAs for which we were unable to identify a target associated with osteoclastogenesis through our search.

FIGURE 8

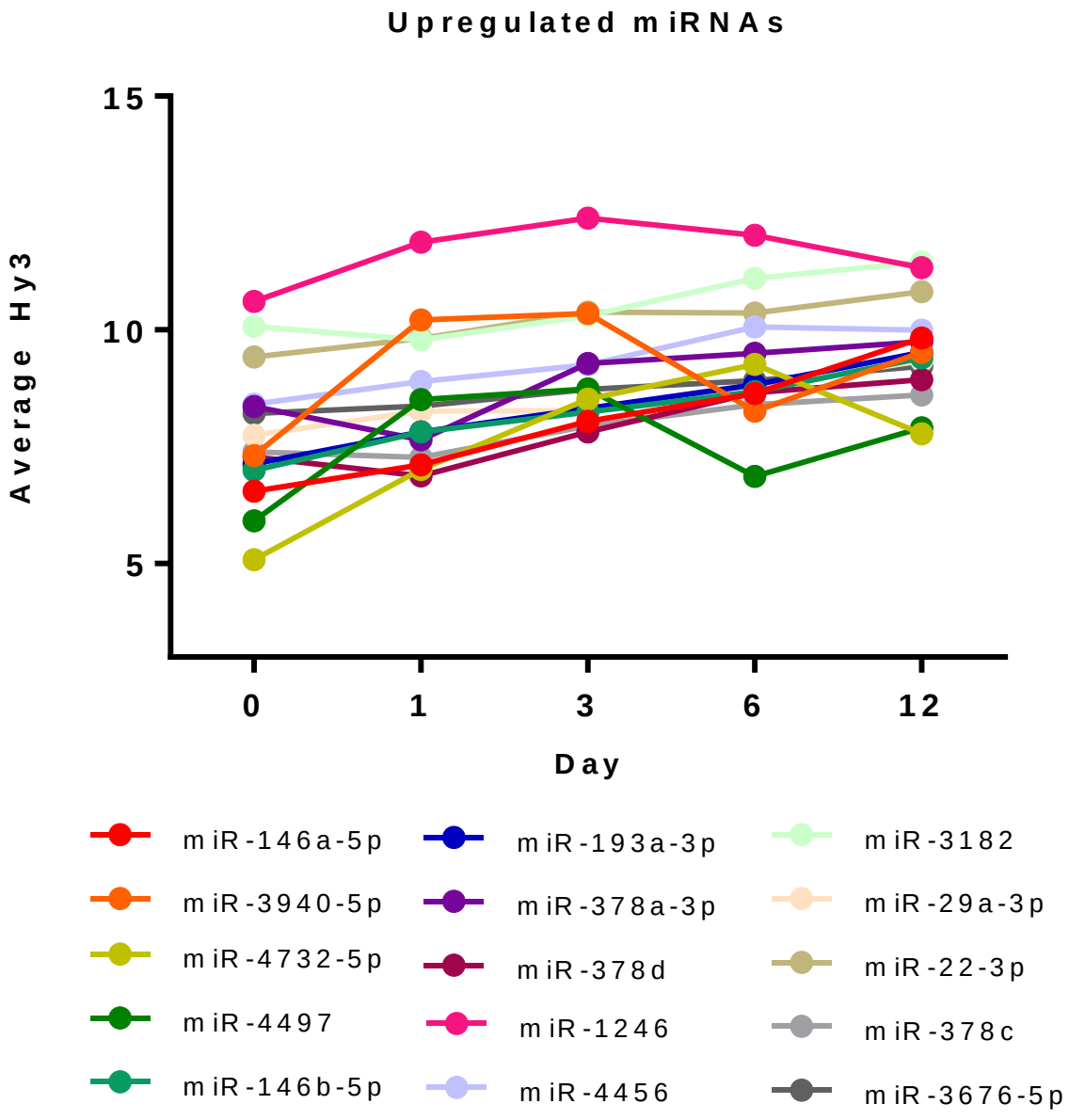

Figure 8: miRNAs that were significantly differentially expressed and up-regulated overall. Chart displays the least square means of expression values at each time point for 4 donors. Note that peak expression levels vary by time point for the miRNAs and that the peak value is not always at Day 12.

FIGURE 9

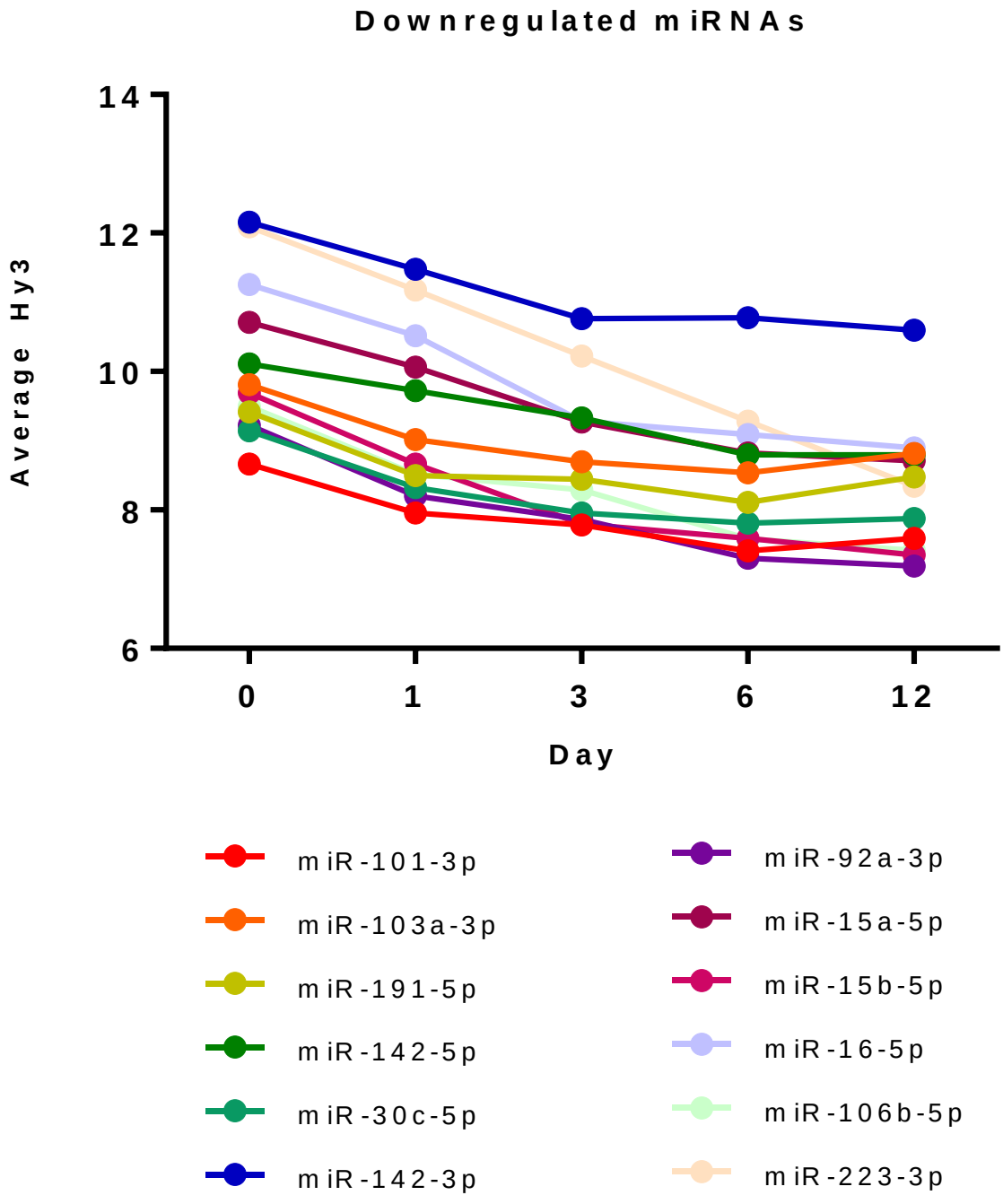

Figure 9: miRNAs that were significantly differentially expressed and down-regulated overall. Chart displays the least square means of expression values at each time point for 4 donors. Note that peak expression levels vary by time point for the miRNAs and that the lowest expression value does not always occur at Day 12.

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